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Bovine PrP^C directly interacts with αB-crystalline

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Abstract **We used a bovine brain cDNA library to perform a yeast two-hybrid assay with bovine mature PrP^C as bait. The screening result showed that αB-crystalline interacted with PrP^C. The interaction was further evaluated both in vivo and in vitro with different methods, such as immunofluorescent colocalization, native polyacrylamide-gel electrophoresis, and IAsys biosensor assays. The results suggested that αB-crystalline may have the ability to refold denatured prion proteins, and provided first evidence that αB-crystalline is directly associated with prion protein.**

Keywords: Protein interaction; Prion; PrP^C; αB-Crystalline; Small heat-shock protein

1. Introduction

Transmissible spongiform encephalopathies (TSE) or prion diseases are an unusual group of fatal neurodegenerative disorders, including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and Creutzfeldt–Jacob disease (CJD) in human. The hallmark of the diseases was an aberrant metabolism and resulting accumulations of scrapie form of prion protein (PrP^{Sc}), which is rich in β-sheets, partial protease resistant and converted from the cellular normal prion protein (PrP^C), which is a soluble form with a predominant α-helical conformation [1]. The molecular mechanism of the conversion still remains enigmatic. Some studies have suggested that other protein may influence TSE pathogenesis or regulate the disease [2,3], but the protein remain unidentified.

PrP^C is a highly conserved GPI-anchored protein and is mainly expressed in neurons [4,5]. However, its physiological function is unclear. Several studies have demonstrated that PrP^C is a copper binding protein, and the copper binding endows PrP^C with superoxide dismutase activity [6,7]. Attempts to gain further insight into the putative function of PrP^C have led to characterization of some related proteins, such as mouse STLI [8,9], Synapsin, Grb-2 and Pint 1 [10], N-CAMs [11,12], Kringle domains [13], immunoglobulin C2 and fibronectin type III-like motifs [14], Pli45 and Pli110, glial fibrillary acidic protein [15], which were associated with murine PrP^C, and 37-kDa/67-kDa LRP [16,17] acting as a human PrP^C receptor. These proteins could interact with PrP^C at the cell membrane, in endocytic compartments or in the secretory pathway, suggesting that PrP^C might play a role in signal transduction [9,10] and in neuronal adhesion and neurite growth [11,12]. But till now, none of interrelated proteins in bovine was found.

With the increased cases of BSE outbreaking, great efforts have been made to explore the disease. In this paper, using a cDNA library derived from bovine brain as prey, we performed a yeast two-hybrid assay with bovine mature PrP^C ($BoPrP^C$) as bait. The results showed that $BoPrP^C$ associated with αB-crystalline. αB-crystalline is a member of the small heat-shock proteins (sHsp) family of molecular chaperones. sHsps generally have the ability to refold stressed proteins [18,19]. Our results revealed that $BoPrP^C$ can directly interact with αB-crystalline.

2. Materials and methods

2.1. Yeast two-hybrid screen

2.1.1. Construction of pGBDKT7-PrP^C. The gene of mature $BoPrP^C$ (25-242) was amplified by polymerase chain reaction (PCR) using bovine genomic DNA as a template and then cloned into pGBDKT7 (Clontech) vector via *Nde*I–*Eco*RI restriction sites, yielding pGBDKT7-PrP^C as bait.

2.1.2. Construction of pGADT7-cDNA. The bovine brain cDNA library (Uni-ZAP XR Library) was purchased from Stratagene, cDNA fragments were ligated into pGADT7 (Clontech) with *Eco*RI–*Xho*I sites, resulting in pGADT7-cDNA as prey.

2.1.3. Yeast two-hybrid screen. *Saccharomyces cerevisiae* strain PJ69-4A cells were co-transformed with both prey and bait plasmids. Positive clones were selected on synthetic dropout (SD) medium in the absence of four nutrients (Leu, Trp, Ade and His). It grew 5–7 days, and was tested for β-galactosidase activity. Colonies turning to blue were reserved, and the positive results were confirmed by repeating the assay. Then the pGADT7-cDNA plamids isolated from the positive clones were co-transformed into yeast with the empty plasmid pGBDKT7 or the bait plasmid pGBDKT7-PrP^C, for the autoactivation test or the interaction reconfirmation, respectively.

The inserted DNA fragments on the positive clones were sequenced with the T7-sequencing primer. Sequences were performed by Blast network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast).

2.2. Immunofluorescent colocalization

BHK21 cells were grown in DMEM medium, containing 10% fetal calf serum. At 75% confluence, cells were co-transfected with 3 μg pSC6-T7-NEO plasmid (kindly provided by Dr. Billeter) expressing

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Abbreviations: TSE, transmissible spongiform encephalopathies; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jacob disease; $BoPrP^C$, bovine mature PrP^C; sHsp, small heat-shock proteins

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recombinant T7 polymerase [20] and 5 μg bait and prey plasmids DNA mixture using Lipofectamine™ 2000 (Invitrogen). Transfection efficiency was evaluated by a green fluorescent protein(GFP) plasmid under the control of T7 promoter. Immunofluorescent staining was performed 48 h after the cotransfection. PrP^C-Myc was recognized by the anti-Myc-FITC monoclonal antibody (Invitrogen), whereas crystalline-HA was recognized by the anti-HA-Rhodamine monoclonal antibody (Roche). The fluorescent signals were analyzed by the Leica TCS-AOBS-SP2-MP laser confocal microscope.

2.3. Protein preparation

The αB-crystalline cDNA was amplified by PCR using a bovine brain cDNA library as template, and was inserted into vector pET30a (Novagen) via *EcoR*I–*Xho*I sites, resulting in pET30a-αB-crystalline plasmid. The method of constructing pET30a-PrP^C is the same as that of pGBDKT7-PrP^C as described above. After verification by sequencing, the two constructs of plasmids were transformed into *E. coli* BL21 (DE3) separately. The on-column purification and refolding of pET30a-PrP^C were performed as described previously [21]. The expressed αB-crystalline was purified using immobilized metal affinity chromatography (MAMC) with Ni-NTA His Bind Resins (Novagen) according to the manufacturer's instructions.

The purified proteins were verified by SDS–PAGE and the concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard according to the manufacturer's instructions.

2.4. IAsys biosensor measurements

The measurements were performed using an IAsys biosensor (Affinity Sensors, Cambridge, UK). $BoPrP^C$ was immobilized on carboxymethyl-dextran cuvette surfaces. Binding assay was performed in 50 mM sodium phosphate, pH 7.2. αB-crystalline protein was added to a final concentration of 6–192 nM in a total volume of 200 μl, and changes in resonant angle were monitored at 1 s intervals for approximately 300 s. The experiments were performed at 25 °C with a stirrer speed of 85 rpm. For repeated measurements with different concentration of αB-crystalline using the same cuvette, the surface was regenerated by washing it two times in 20 mM $NaHCO_3$.

2.5. Native polyacrylamide-gel electrophoresis

The action mixtures of $BoPrP^C$ and αB-crystalline protein at indicated concentrations were mixed with glycine native sample loading buffer and were then loaded (25 μl per well) on a glycine precast gel (7.5%). Gel electrophoresis was carried out with constant 150 V at 4 °C for 1.5 h.The gel was then stained with Coomassie blue.

2.6. Refold assays of PrP

The BoPrP was unfolded in 8 M Urea, 100 mM sodium phosphate, 10 mM β-mercaptoethanol, 0.5% Triton-100 and 10 mM Tris–HCl solution at pH 8.0 overnight at 4 °C. Aliquots of samples (3 mg/ml) would be assayed for refolding. The mixture was diluted 100 times with 100 mM sodium phosphate, 10 mM Tris–HCl solution (pH 7.0) containing a diverse concentrations of αB-crystalline (0, 1, 2, 3, 4, 5 μg/ml). The diluted solutions were centrifuged for 5 min at $12000 \times g$, the suspensions were concentrated by centrifugal filter devices (Millipore), and then analyzed by SDS–PAGE. At the same time, the conformation of the suspensions was investigated by JASCO J-810 Spectropolarimeter. The circular dichroism spectra gave its raw output in ellipticity, and was measured in millidegrees.

3. Result

To find proteins physically interacting with the bovine cellular prion protein, we employed a yeast two-hybrid screening method. The screening of the library revealed that several yeast clones had positive signals in the β-galactosidase assay. The pGADT7-cDNA plasmids isolated from positive clones were transformed into *E. coli* for amplification. After isolation from *E. coli*, the positive pGADT7-cDNA was co-transformed with pGBKT7 or pGBDKT7-PrP^C for the autoactivation test or for the reconfirmation of the interaction. Sequencing of the isolated positive plasmids showed that several of these positive clones' sequences were identical with the bovine αB-crystalline protein gene sequence. Fig. 1(A) shows that αB-crystalline was not of auto-activity and Fig. 1(B) shows that αB-crystalline interacted with $BoPrP^C$ by β-galactosidase assay.

To determine whether $BoPrP^C$ and αB-crystalline were co-localized in mammalian cells, pGADT7-αB-crystalline, pGBDKT7-$BoPrP^C$ expression vectors were co-transformed into BHK21 cells in which the co-transformed pSC6-T7-NEO plasmid provided T7 polymerase. The expression of $BoPrP^C$-Myc protein and αB-crystalline-HA protein was monitored by immunofluorescent staining using anti-Myc-FITC monoclonal antibody and anti-HA-Rhodamine monoclonal antibody, respectively. Confocal microscope analysis of fluorescent image revealed that $BoPrP^C$ and αB-crystalline proteins were co-localizing in mammalian cells (Fig. 2). The bait plasmid that encoded the mature form of $BoPrP^C$ that was located in the cytosol, so $BoPrP^C$ and αB-crystalline were co-localized in the cytosol.

Native polyacrylamide-gel electrophoresis (N-PAGE) and IAsys biosensor assays were employed to detect the association of the two proteins in vitro. In the N-PAGE (Fig. 3), $BoPrP^C$, with net positive charges, migrated up and off the gel, so no band could be detected in the gel; whereas αB-crystalline migrated down in the gel, for its net negative charge. The mixture of $BoPrP^C$ and αB-crystalline showed two bands. The upper band had the same position as isolated αB-crystalline, and

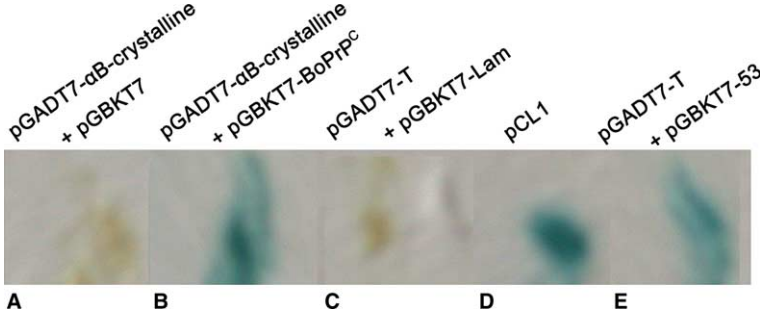

Fig. 1. Identification of interaction between $BoPrP^C$ and αB-crystalline in the yeast two-hybrid system by assaying β-galactosidase activities. (A) No auto-activity was found in pGADT7-αB-crystalline, (B) interaction of $BoPrP^C$ with αB-crystalline, (C) negative control, human lamin C did not interact with SV40 large T-antigen, (D) positive control, pCL1 encoded full-length, wild-type GAL4 protein, (E) positive control, murine P53 interacted with SV40 large T-antigen.

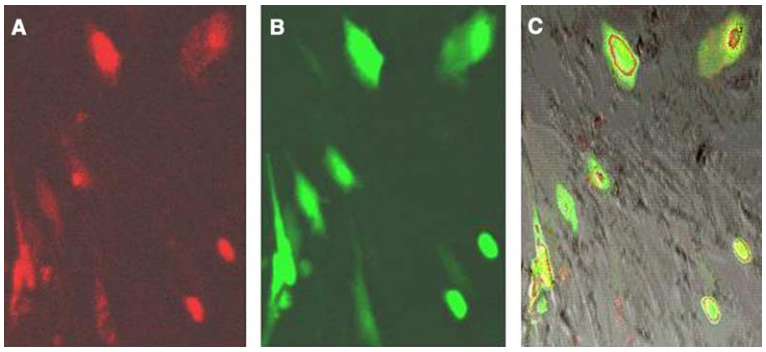

Fig. 2. BoPrP^C co-localized with αB-crystalline in BHK21 cells. (A) Texas red immunofluorescent staining of αB-crystalline-HA fusion protein by monoclonal antibody recognizing HA sequence; (B) FITC immunofluorescent staining of BoPrP^C-Myc fusion protein by monoclonal antibody recognizing the Myc sequence; (C) overlay of FITC and Texas red immunofluorescent staining detected by laser confocal microscope.

the lower one was expected to be the complex formed by the association of BoPrP^C and αB-crystalline (Fig. 3A). In Fig. 3B, the concentrations of BoPrP^{C}are constant, the intensity of the mixture bands increased with increasing concentrations of αB-crystalline (from lane 2 to lane 6). These data showed that formation of the complex depended on the concentration of αB-crystalline.

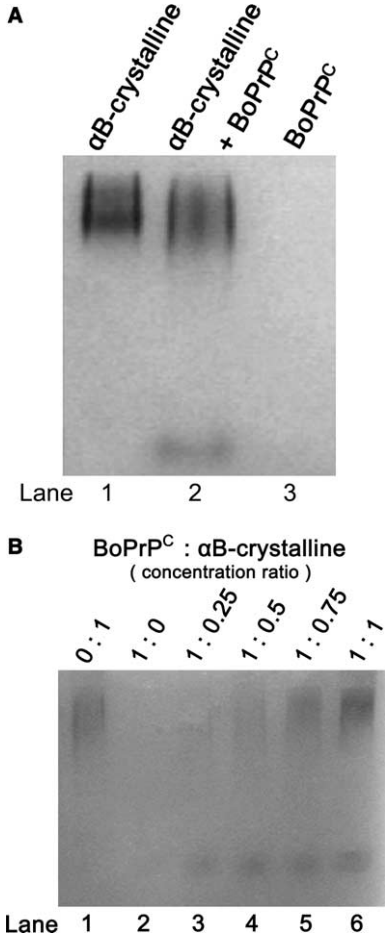

Fig. 3. The associations of BoPrP^C with αB-crystalline were determined by N-PAGE. (A) Lane 1, only loaded αB-crystalline with net negative charge. Lane 2, the reaction complex of BoPrP^C and αB-crystalline showed two bands. The upper band had the same position as isolated αB-crystalline, and the lower one was expected to be the complex of BoPrP^C and αB-crystalline. Lane 3, BoPrP^C, carrying net positive charges, showed no band. (B) With increasing concentrations of αB-crystalline (from lane 2 to lane 6: 0, 30, 60, 90, 120 μM), the intensity of the complex bands increased, while BoPrP^C is at a constant concentration (120 M).

The IAsys biosensor was used to analyze the interaction between the two proteins in real time in vitro. Binding curves are shown in Fig. 4A and indicate the specific binding of the αB-crystalline to immobilized BoPrP^C. The association (k_{ass}) and dissociation constant (k_{diss}) values were calculated based on the K_{on} value for different concentration of αB-crystalline at 6, 12, 24, 48, 96 and 192 nM using the IAsys Fit program algorithm. The k_{ass} and k_{diss} were 671762.65681 M^{-1} S^{-1} and 0.26703 S^{-1}, respectively. From the ratio of the respective rate constants, $K_D = 3.98e-7$ M was determined, the data suggested their binding was high affinity. We also noticed that in Fig. 4(A), the binding curves with 192, 96 and 48 nM differed from those obtained at 24, 12 and 6 nM due to the spontaneous conformation changes in PrP^C under the measuring and regeneration conditions. This accorded with the description of Leclerc et al. [22] that under physiological solvent conditions, monomeric PrP^C on the sensor chip surface was an intrinsically unstable molecule prone to conformational rearrangement.

Fig. 4B fitted the biphasic binding of the IAsys Fit program algorithm, which indicated that BoPrP^C had multiple binding sites of αB-crystalline. The first association, when BoPrP^C was recognized by αB-crystalline, was very fast. Following that process, the BoPrP^C might have also explored the second association site, which was consistent with the Stockel's hypothesis [23] that some factor that affected PrP conversion would have multiple binding sites allowing interactions with both the N-terminal segment and the core domain of PrP.

These findings confirmed that PrP^C could specifically interact with αB-crystalline both in vivo and in vitro.

As a molecular chaperone, αB-crystalline can capture chemically denatured model substrates and prevent their precipitation in a sense [18]. Here, we were interested in whether αB-crystalline had the ability to refold denatured BoPrP. At room temperature, BoPrP was unfolded in 8 M urea and did not significantly refold to a soluble state when diluted into a solution containing 100 mM sodium phosphate, 10 mM Tris–HCl, and pH 7.0. However, when urea-unfolded BoPrP was diluted in the same solution containing different concentration of αB-crystalline, as Fig. 5A shows, with an increase amount of

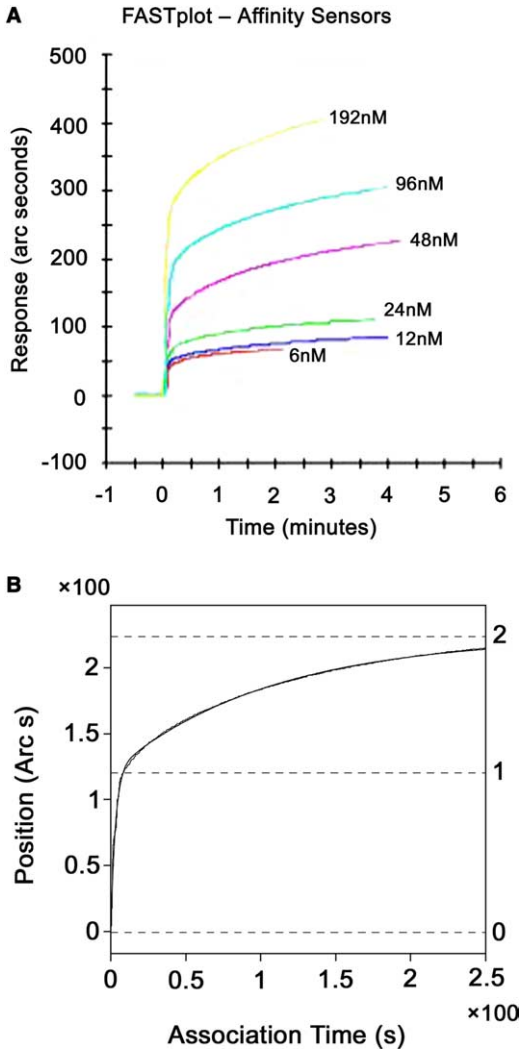

Fig. 4. BoPrP^C-αB-crystalline interaction dynamics was analyzed by an IAsys biosensor. BoPrP^C was immobilized on a carboxylate cuvette. (A) Various concentrations of αB-crystalline were injected into the cuvette equilibrated with a running buffer (50 mM sodium phosphate, pH 7.2), and signals representing the binding of two proteins were monitored. The surface was regenerated with 20 mM $NaHCO_3$ and the binding determination was repeated. (B) Fit of the biphasic binding of the IAsys Fit program algorithm.

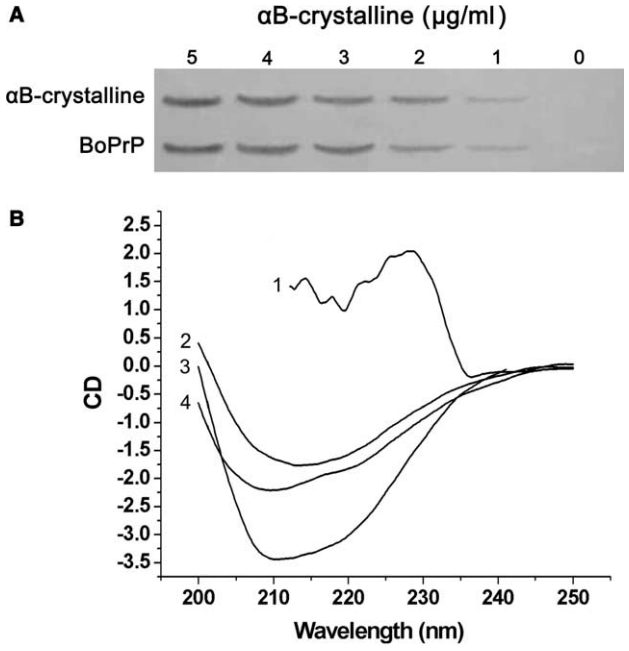

Fig. 5. (A) Refold assays of BoPrP with the help of αB-crystalline. Denatured BoPrP was diluted from 8 M urea into the dilution buffer (100 mM sodium phosphate, 10 mM Tris–HCl, and pH 7.0) containing different concentration of αB-crystalline (0, 1, 2, 3, 4, 5 μg/ml), and the refolded BoPrP presenting in suspension were analyzed by SDS–PAGE. (B) Conformation assays of refold BoPrP by circular dichroism spectra. (The concentrations of each measure samples were 0.3 mg/ml.) Curve 1: BoPrP in 8 M urea was fully unfolded. Curve 2: αB-crystalline in the dilution buffer showed one negative maximum at 216 nm, representing typical β-sheet. Curve 3: BoPrP^C purified in MAMC and refolded on-column showed a negative maximum at 208 nm and a shoulder at 222 nm, indicating BoPrP^C contained α-helix structure. Curve 4: With the help of αB-crystalline, the refolded BoPrP presenting in suspension showed a negative maximum at 208 nm and a shoulder at 222 nm, similar to curve 3.

αB-crystalline, the quantity of soluble BoPrP in the suspensions also increased. And the far-UV CD spectrum of refolded soluble BoPrP displayed a negative maximum at 208 nm and a shoulder at 222 nm (Fig. 5B), suggesting the presence of a significant amount of α-helix. It implied that αB-crystalline could refold denatured BoPrP.

4. Discussion

It is the first time that the binding of BoPrP^C with αB-crystalline was confirmed. αB-Crystalline, which is a member of the sHsp family of molecular chaperones, can recognize unfolded or partially denatured proteins by their hydrophobic surface [18,24], and PrP^C differs markedly from most other globular proteins in that its native state contains a large segment (residues 23–126) that is unstructured in solutions [24–27] and is readily recognized by chaperons. From above results, we also could propose that BoPrP^C directly binded to αB-crystalline, their association could not involve additional factors. Our results also suggested that the association of BoPrP^C and αB-crystalline had multiple binding sites. Our future study will be interested in whether αB-crystalline firstly recognize the unstructured segment of the BoPrP^C by its hydrophobic surface.

Although, αB-crystalline has little expression outside lens under normal conditions, it has been found in many additional tissues under stress situations, such as cardiac, skeletal muscles and brain [28–32]. αB-Crystalline in the neurons of the brain appeared to be closely related to various neurological diseases, and several researches had already showed the association of increased levels of αB-crystalline with these diseases, such as Alexander's disease [18], CJD [31], Alzheimer's disease [32], and Parkinson's disease [33]. The significant feature of these diseases is the amyloid aggregation and the ordered fibril formation may represent of off-pathway amorphous aggregation. sHsps acting as molecular chaperones generally prevent the aggregation of other proteins under conditions of physiological stress [18,34]. And they exhibited a cytoprotective function, preventing neuronal cell

death under various noxious conditions. Rekas et al. [35] suggested that αB-crystalline redirected a-synuclein from a fibril-formation pathway towards an amorphous aggregation pathway, and reduced the amount of physiologically stable amyloid deposits. However, Kudva et al. [36] suggested αB-crystalline prevented in vitro fibril formation of Aβ1-42, and enhanced amyloid neurotoxicity. Whether the association between $BoPrP^C$ and αB-crystalline can prevent the PrP from fibril and neurotoxicity still remains unknown. Our future study will focus on this. Our data firstly confirm αB-crystalline directly interacts with $BoPrP^C$. It may be a key step for both the understanding of BSE pathogenesis and the development of therapeutics.

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